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ORIGINAL PAPER

Distribution of the type III effector proteins-encoding genes among nosocomial *Pseudomonas aeruginosa* isolates from Bulgaria

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Abstract The aim of this study was to determine the prevalence of type III effector proteins (ExoS, ExoU, ExoT and ExoY)-encoding genes among clonally unrelated nosocomial *Pseudomonas aeruginosa* strains and to analyze their distribution in respect to the infection site and antimicrobial resistance. Polymerase chain reaction-based detection of the genes was performed on 176 non-duplicate *P. aeruginosa* isolates from three University hospitals in Sofia, previously genotyped by random amplified polymorphic DNA technique. The prevalence of the studied genes was as follows: *exoS*–61.9%, *exoU*–32.4%, *exoT*–100%, and *exoY*–85.8%. The part of *P. aeruginosa* strains harboring either the *exoS* (54.0%) or the *exoU* (23.8%) gene was higher ($P<0.001$) than that of isolates containing both genes (8.5%). The gene dissemination varied according to the infection localization. The *exoU* gene manifested a higher spread ($P<0.001$) among multidrug-resistant (MDR) than in non-MDR strains (42.6 vs 18.7%). In conclusion, the *P. aeruginosa* type III secretion system is present in nearly all studied isolates but the individual isolates from distinct infection sites differ in their effector genotypes. The ubiquity of type III effector proteins-encoding genes among clinical isolates is consistent with an important role for this system in *P. aeruginosa* pathogenesis.

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Introduction

Pseudomonas aeruginosa is a major cause of hospital-acquired infections and the most significant pulmonary pathogen in cystic fibrosis patients (Pinheiro et al. 2008; Rajan and Saiman 2002). *P. aeruginosa* is responsible for 10–15% of nosocomial infections worldwide (Blanc et al. 1998). It is not surprising that these illnesses are associated with significant morbidity and mortality due to the organism's capacity to adapt easily to changes in the environment, to rapidly develop resistance to antibiotics, and to produce an arsenal of virulence factors (Strateva 2008).

To cause such a severe disease, *P. aeruginosa* utilizes a large number of secreted and cell-associated virulence factors (Van Delden and Iglewski 1998). An important and recently recognized virulence determinant is the complex type III secretion system which injects effector proteins into host cells (Hauser 2009). The genes encoding the secretion, translocation and regulatory machinery of this system are clustered together in the 55-min region of the *P. aeruginosa* chromosome (http://www.pseudomonas.com).

In contrast to the clustered genes encoding the type III transport machinery, the genes encoding the type III effector proteins appear to be scattered throughout the chromosome (Stover et al. 2000). To date, four effector proteins have been identified: ExoS (exoenzyme S), ExoU (exoenzyme U), ExoT (exoenzyme T) and ExoY (exoenzyme Y) (Engel and Balachandran 2009). ExoS and ExoT are closely related bifunctional proteins with N-terminal GTPase-activating protein activity toward Rho family

proteins and C-terminal ADP ribosylase activity toward distinct and non-overlapping set of targets (Goehring et al. 1999; Krall et al. 2000). ExoY is an adenylate cyclase that increases intracellular levels of cAMP (Yahr et al. 1998). Intoxication with ExoS, ExoT and ExoY causes cell rounding and detachment and may contribute to infection by inhibiting or preventing bacterial uptake and phagocytosis (Vallis et al. 1999). ExoU possesses phospholipase A_2 and lysophospholipase activities (Phillips et al. 2003; Tamura et al. 2004) that lead to rapid necrotic death in many cell types (Finck-Barbancon et al. 1997; Vallis et al. 1999). It is also associated with accelerated lung injury, and plays a role in the development of septic shock (Allewelt et al. 2000; Kurahashi et al. 1999).

Interestingly, the genes encoding some *P. aeruginosa* type III effector proteins are found in some isolates but not in others (Feltman et al. 2001). The distribution of these genes amongst clinical isolates of *P. aeruginosa* remains to be elucidated. Furthermore, the frequency of effector genes in populations of isolates from different disease sites has not been thoroughly examined. For that reason, the aim of the present study was to determine the prevalence of the type III effector proteins-encoding genes among nosocomial *P. aeruginosa* isolates and to analyze the values in respect to the infection localization and antimicrobial resistance.

Materials and methods

Bacterial isolates A total of 176 nosocomial isolates of *P. aeruginosa* was used in the present study. They were cultured during 2001–2009 from hospitalized patients (n=176) of different types of ward in three University hospitals in Sofia and were obtained as follows: from urine (n=67), tracheal aspirates (n=26), bronchial lavage (n=12), sputum (n=12), pleural fluid (n=2), surgical wounds or abscesses (n=29), blood (n=8), nose (n=9), throat (n=8), and bile (n=3). Bacterial identification was performed using a BBL Enteric/Nonfermenter ID system (Becton Dickinson).

Definition of multidrug-resistant (MDR) isolates MDR *P. aeruginosa* isolates were defined as resistant to one or more representatives of at least three antibiotic classes with antipseudomonal activity (β-lactams, aminoglycosides and fluoroquinolones) (Wang et al. 2006; Pagani et al. 2005; Kirikae et al. 2008), using the conventional serial agar dilution method. The minimal inhibitory concentrations were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) 2007 guide-line (CLSI 2007).

DNA isolation Bacterial DNA isolation was performed from a single colony on Brucella sheep blood agar using the illustra bacteria genomicPrep Mini Spin Kit (GE Healthcare) according to the manufacturer's guidelines. The DNA concentration range was about 8 μg/ml.

Polymerase chain reaction (PCR) amplification of the type III effector proteins-encoding genes The genes were amplified with specific primers (Alpha DNA) listed in Table 1. PCR was carried out with 2 μl template DNA, 0.25 μM of each primer, 0.2 mM deoxyribonucleoside triphosphates, 1× reaction buffer, 2 mM $MgCl_2$ and 1.5 U Prime *Taq* DNA polymerase (GeNet Bio) in a total volume of 25 μl. The DNA was amplified using the following protocol: initial denaturation (94°C for 5 min), followed by 25–30 cycles of denaturation (94°C for 35–45 s), annealing (58–64°C, from 45 s to 50 s), and extension (72°C, from 45 s to 1 min 30 s), with a single final extension of 7 min at 72°C. PCR products were separated in 1.5% agarose gel for 50–110 min at 130 V, stained with ethidium bromide (0.5 μg/ml) and detected by UV transillumination (wavelength 312 nm). Amplified genes were identified on the basis of fragment size (also shown in Table 1).

DNA sequencing Selected *exoS* (n=10) and *exoU* (n=10) PCR products amplified from different *P. aeruginosa* isolates were purified by an ExoSAP-IT reagent (Amersham Biosciences). Sequencing reactions were performed using the sequencing primers listed in Table 1 (Strateva 2008), the primers for amplification and a BigDye terminator v3.1 kit (Applera) in an automated sequencer (ABI 310 sequence genetic analyzer; Applied Biosystems). The nucleotide and deduced amino acid sequences were analyzed with software available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The GenBank accession numbers of the published sequences are: **AY029250** (*P. aeruginosa exoS* gene) and **U97065** (*P. aeruginosa* ExoU operon).

Random amplified polymorphic DNA (RAPD) analysis - RAPD was performed with Ready-To-Go RAPD Analysis Beads (GE Healthcare) according to the manufacturer's guidelines. The amplifications were carried out with 2 μl DNA and 1 μl primer (RAPD-4 5′-AAGAGCCCGT-3′) in a total volume of 25 μl. The following protocol was applied: 1 cycle of 5 min at 95°C, followed by 45 cycles of denaturation for 1 min at 95°C, annealing of primers for 1 min at 36°C ,and elongation for 2 min at 72°C. The amplifications products were compared by electrophoresis of 10-μl samples in 2% agarose gel stained with ethidium bromide and photographed under UV light. The bends generated were analyzed using Dice coefficient (similarity coefficient, S_{AB}) for every pair of isolates. Isolates with S_{AB} value of >70% are considered to be closely related. Two *E. coli* strain (BL21 (DE3) and C1a) DNAs were used

Table 1 Primers used for amplification and sequencing of the type III effector proteins-encoding genes

Primers	Target gene	Sequence (5′–3′)	Product size (bp)	Position on *P. aeruginosa* PAO1 chromosome (locus)	Annealing temperature (ºC)	Source
Pairs for PCR						
exoS-F	*exoS*	CTT GAA GGG ACT CGA CAA GG	504	PA3841	60	Lanotte et al. 2004
exoS-R		TTC AGG TCC GCG TAG TGA AT				
exoT-F	*exoT*	CAA TCA TCT CAG CAG AAC CC	1159	PA0044	58	Finnan et al. 2004
exoT-R		TGT CGT AGA GGA TCT CCT G				
exoY-F	*exoY*	TAT CGA CGG TCA TCG TCA GGT	1035	PA2191	64	Finnan et al. 2004
exoY-R		TTG ATG CAC TCG ACC AGC AAG				
exoU-F	*exoU*	GGG AAT ACT TTC CGG GAA GTT	428	PS14 within *P. aeruginosa* PA14 pathogenicity island PAPI-2	60	Allewelt et al. 2000
exoU-R		CGA TCT CGC TGC TAA TGT GTT				
Sequencing primers						
exoS-F-seq	*exoS*	ATG CAT ATT CAA TCG CTT CA				Strateva 2008
exoS-R-seq	*exoS*	CGA CCG GTC AGG CCA GAT				Strateva 2008
exoU-R-seq1	*exoU*	TCA TGT GAA CTC CTT ATT				Strateva 2008
exoU-R-seq2	*exoU*	CGA GAG AAG CGA AGG TAT GA				Strateva 2008

PCR Polymerase chain reaction, *F* forward, *R* reverse

exoS exoenzyme S-encoding gene, *exoT* exoenzyme T-encoding gene, *exoY* exoenzyme Y-encoding gene, *exoU* exoenzyme U-encoding gene

as controls to assay the ability of the RAPD beads to amplify DNA and identify polymorphisms. The control DNAs were genotyped by both RAPD-4 and RAPD-2 (5′-GTTT CGCTCC-3′) primers.

Statistical analysis The distribution of virulence genes with respect to isolate origin was compared using Student's *t* test. A *P* value below 0.05 was considered to be statistically significant.

Results and discussion

Evaluation of clonal relatedness of the studied *P. aeruginosa* isolates by RAPD typing

A total of 176 *P. aeruginosa* nosocomial strains isolated during an 8-year period were analyzed by RAPD typing. RAPD fingerprints demonstrated that most strains were of distinct genotype and determined the presence of 106 RAPD patterns. A large number of bands (with size from 130 to 2,200 bp) were generated by the RAPD-4 primer. Eleven strains revealed unique RAPD profiles. A few strains were closely related (S_{AB}>70%)-9 nosocomial *P. aeruginosa* isolates from 4 different clinics of the University hospital No 1 (with S_{AB} value of 82%-Fig. 1), 14 isolates from the Neonatology clinic (86%) and 8 isolates from the Urology clinic (82.6%) of the University hospital No 2. In summary, the RAPD analysis confirmed the significant diversity of the strains included in the present study.

Overall prevalence of the type III effector proteins-encoding genes

The frequencies of occurrence of these genes detected by PCR in all studied strains (n=176) were as follows: *exoS*–61.9%, *exoU*–32.4%, *exoT*–100%, and *exoY*–85.8%. The sequences of the *exoS* and *exoU* genes amplified from different isolates were identical for all examined isolates and 100% identical to the known sequences (AY029250 and U97065, respectively). The prevalence of three genes (*exoS*, *exoU* and *exoY*) was similar to that ascertained by Feltman et al. (2001), respectively 72, 28 and 89%, among clinical *P. aeruginosa* isolated in USA during 1999–2000. The absolute presence of *exoT* was not surprising. Several recent studies reported a very high prevalence of this gene among clinical and environmental isolates of *P. aeruginosa* (Feltman et al. 2001; Lomholt et al. 2001; Winstanley et al. 2005).

The majority of the 176 strains included in our study (47.2%) exhibited a combination of *exoS*, *exoT* and *exoY*; 21% possessed a combination of *exoU*, *exoT* and *exoY* (Table 2). The part of *P. aeruginosa* strains containing either the *exoS* (54.0%) or the *exoU* gene (28.3%) was significantly higher (P<0.001) than the part of isolates harboring both genes *exoS*+*exoU* (8.5%). Previously, Feltman et al. (2001) reported that of 115 *P. aeruginosa* clinical and environmental

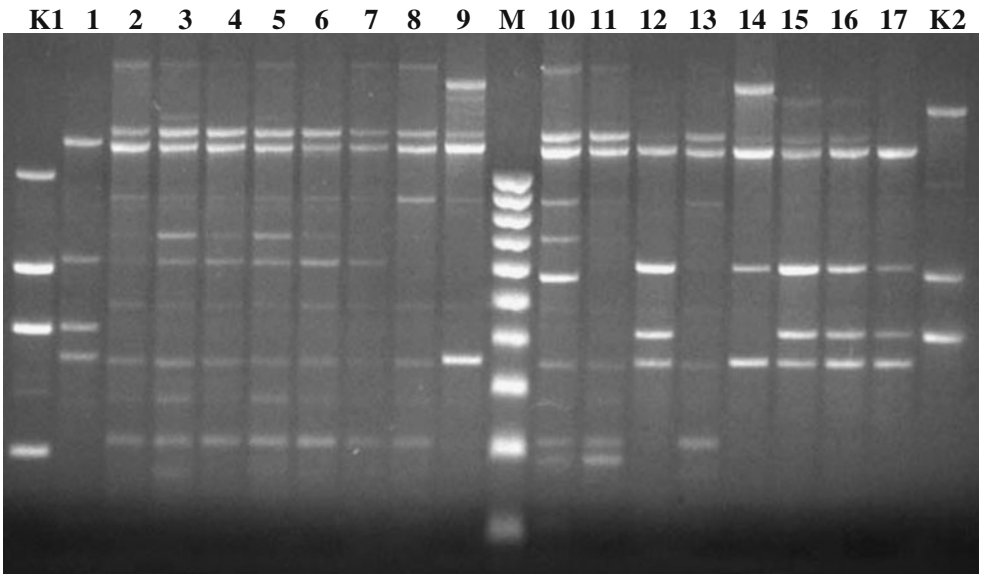

Fig. 1 Randomly amplified polymorphic DNA (RAPD) of *P. aeruginosa* isolates generated with RAPD-4 primer. *Lanes*: *K1* RAPD profile of the reference strain *E. coli* BL21 (DE3) generated with RAPD-2 primer, *K2* RAPD profile of the reference strain *E. coli* C1a generated with RAPD-2 primer, *M* standard size marker (100-bp ladder), *1–17* nosocomial *P. aeruginosa* isolates. *Lanes 2–8, 10* and *11* show the RAPD profiles of nine closely related strains (S_{AB} value of 82%) from four different clinics

isolates, 82 contained the *exoS* but not the *exoU* gene, 31 contained the *exoU* but not the *exoS* gene and a single contained both genes. Two recent studies demonstrated a very low frequency of occurrence of *P. aeruginosa* blood-stream isolates harboring both genes, respectively 2.2 and 1.6% (Berthelot et al. 2003; Garey et al. 2008). Winstanley et al. (2005) established a similar find among *P. aeruginosa* isolates associated with ulcerative keratitis. In a recent multicenter study conducted by Pirnay et al. (2009), 72.6% of all *P. aeruginosa* isolates harbored the *exoS* gene, 23.1% contained the *exoU* gene and with the exception of three strains, the carriage of *exoU* and *exoS* was mutually exclusive. In contrast, Finnan et al. (2004) found 75% dissemination of both genes in clinical and environmental *P. aeruginosa* isolates.

Prevalence of the type III effector proteins-encoding genes with respect to the infection localization

The distribution of virulence genes encoding the type III effector proteins varied in respect to the infection localization (Table 3).

(1) The presence of *exoS* was the highest among *P. aeruginosa* isolates from blood (87.5%) and significantly different only with those obtained from in-patients with lower respiratory tract infections (LRTIs) (53.8%)-$P<0.02$.
(2) The *exoU* frequencies were the most expressive in the wound (51.7%) and LRTIs *P. aeruginosa* isolates (40.4%). They were significantly higher than those of the isolates from urine (19.4%), $P<0.01$ and $P<0.02$, respectively, and blood (12.5%), $P<0.01$ and $P<0.05$, respectively.
(3) The *exoY* gene was disseminated among all studied isolates from blood, which was higher than the *exoY*-distribution in *P. aeruginosa* isolates from urine (85.1%, $P<0.001$), LRTIs (82.7%, $P<0.01$) and wound (82.8%, $P<0.02$).

The prevalence of the *exoS* among our LRTIs-isolates (53.8%) was lower than that in LRTIs *P. aeruginosa* isolates from the studies mentioned above (Lanotte et al. 2004, 80%; Feltman et al. 2001, 75%). ExoS is responsible for direct tissue destruction in lung infection and may be important for bacterial dissemination (Nicas et al. 1985a; b).

In this study, the distribution of the *exoU* gene was very heterogeneous. The gene was found predominantly in *P. aeruginosa* isolates from LRTIs and wounds. Its prevalence in our wound isolates (51.7%) was similar to that ascertained by Feltman et al. (2001) in the USA (40.0%). In contrast, Lomholt et al. (2001) reported an absence of the

Table 2 Distribution of the *exo* gene patterns among nosocomial *P. aeruginosa* isolates (n=176)

Gene pattern	Number (%) of isolates
$exoS^-/exoU^-/exoT^+/exoY^-$	4 (2.3)
$exoS^+/exoU^-/exoT^+/exoY^-$	12 (6.8)
$exoS^-/exoU^+/exoT^+/exoY^-$	5 (2.8)
$exoS^-/exoU^-/exoT^+/exoY^+$	20 (11.4)
$exoS^+/exoU^-/exoT^+/exoY^+$	83 (47.2)
$exoS^-/exoU^+/exoT^+/exoY^+$	37 (21.0)
$exoS^+/exoU^+/exoT^+/exoY^-$	3 (1.7)
$exoS^+/exoU^+/exoT^+/exoY^+$	12 (6.8)

exo Genes encoding type III effector proteins, + presence, − absence

Table 3 Prevalence (as percentage) of the type III effector proteins-encoding genes in nosocomial *P. aeruginosa* isolates, in respect to the infection site

Gene	Isolate origin					
	Urine (n=67)	LRTIs (n=52)	URTIs (n=17)	Wounds (n=29)	Blood (n=8)	Total[a] (n=176)
exoS	62.7	53.8	70.6	62.1	87.5	61.9
exoU	19.4	40.4	35.3	51.7	12.5	32.4
exoT	100.0	100.0	100.0	100.0	100.0	100.0
exoY	85.1	82.7	94.1	82.8	100.0	85.8

LRTIs Lower respiratory tract infections, *URTIs* upper respiratory tract infections, *exoS* exoenzyme S-encoding gene, *exoU* exoenzyme U-encoding gene, *exoT* exoenzyme T-encoding gene, *exoY* exoenzyme Y-encoding gene

[a] Frequency of the type III effector gene in all studied isolates of *P. aeruginosa*, including three bile isolates

exoU in a small series of 11 wound isolates of *P. aeruginosa* (Lomholt et al. 2001). Our urine isolates showed a low percentage of *exoU* carriers (19.4%), while Lomholt et al. did not find urine isolates harboring the *exoU* gene.

It is known that 90% of ExoU-producing *P. aeruginosa* strains are associated with severe infections (Hauser et al. 2002). Of the type III secretion proteins, ExoU is the most cytotoxic. Its secretion is a marker for highly virulent *P. aeruginosa* isolates obtained from patients with hospital-acquired pneumonia (Schulert et al. 2003).

Prevalence of the type III effector proteins-encoding genes with respect to the antimicrobial resistance

Of the 176 nosocomial *P. aeruginosa* isolates, 101 (57.4%) were MDR. One of the genes, *exoU* manifested a significantly higher spread (P<0.001) among MDR than in non-MDR strains of *P. aeruginosa* (42.6 vs 18.7%) (Fig. 2). Twelve isolates contained the four studied genes (Table 2), moreover 8 of them were MDR.

In a recent accomplished study, Garey et al. (2008) determined the prevalence of *exoU* and *exoS* from bloodstream isolates of hospitalized patients with *P. aeruginosa* bacteremia and ascertained that the isolates containing the *exoU* gene were significantly more resistant (P<0.05) to different classes of antimicrobials: β-lactams (piperacillin/tazobactam, ceftazidime, cefepime, carbapenems), fluoroquinolones, and aminoglycosides (gentamicin). Other research suggested that the multidrug resistance in ocular *P. aeruginosa* was more commonly associated with strains having cytotoxicity and *exoU* gene, and belonging to serotype E (Zhu et al. 2006). Recently, Zaborina et al. (2006) screened consecutively isolated MDR *P. aeruginosa* clinical strains for their ability to disrupt the integrity of human cultured intestinal epithelial cells (Caco-2) and correlated these findings to related virulence phenotypes such as adhesiveness, motility, biofilm formation and cytotoxicity. These strains were characterized and found to harbor the *exoU* gene and to display high swimming motility and adhesiveness.

Recently, it was reported the worldwide spread and persistence of MDR clone comprising *P. aeruginosa* O12 isolates which exhibited the *exoS*$^+$/*exoU*$^-$ genotype (Pirnay et al. 2009). Moreover, most MDR clinical isolates of *P. aeruginosa* reveal either O11 or O12 serotype (Sekiguchi et al. 2007; Pirnay et al. 2009). Evaluation of the spread of these serotypes among Bulgarian nosocomial MDR *P. aeruginosa* strains, as well as their corresponding effector genotypes, should be objectives of our future investigations.

Whether MDR *P. aeruginosa* strains necessarily express a more virulent phenotype continues to remain a controversial issue (Di Martino et al. 2002).

In conclusion, our results suggest that type III secretion genes are found in nearly all investigated *P. aeruginosa* but that individual isolates from distinct infection sites differ in their effector genotypes. Our data confirm that the *exoU*

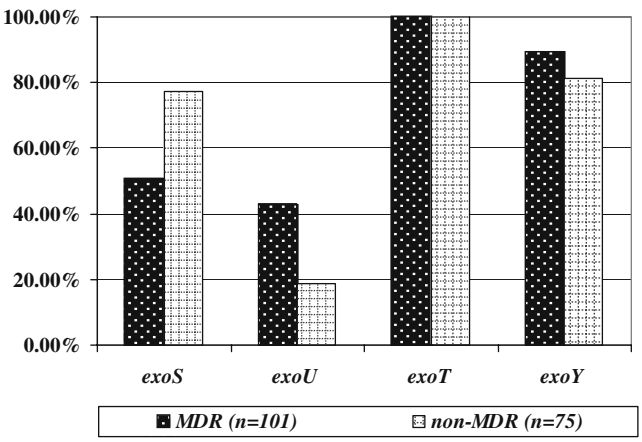

Fig. 2 Prevalence (as percentages) of the type III effector proteins-encoding genes among MDR and non-MDR *P. aeruginosa* isolates from non-cystic fibrosis patients. *MDR* multidrug-resisrant, *exoS* exoenzyme S-encoding gene, *exoU* exoenzyme U-encoding gene, *exoT* exoenzyme T-encoding gene, *exoY* exoenzyme Y-encoding gene

and *exoS* genes are almost mutually exclusive. Nosocomial isolates of *P. aeruginosa* harboring the *exoU* gene were more likely to be resistant to multiple antibiotics. The ubiquity of type III effector proteins-encoding genes among clinical isolates is consistent with an important role for this system in human disease. Future studies to determine the prevalence of clinical isolates capable of secreting specific effector proteins and whether secretion correlates with infection prognosis are necessary for evaluation of the type III secretion system in *P. aeruginosa* pathogenesis.

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The authors declare that they have no conflict of interest.

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